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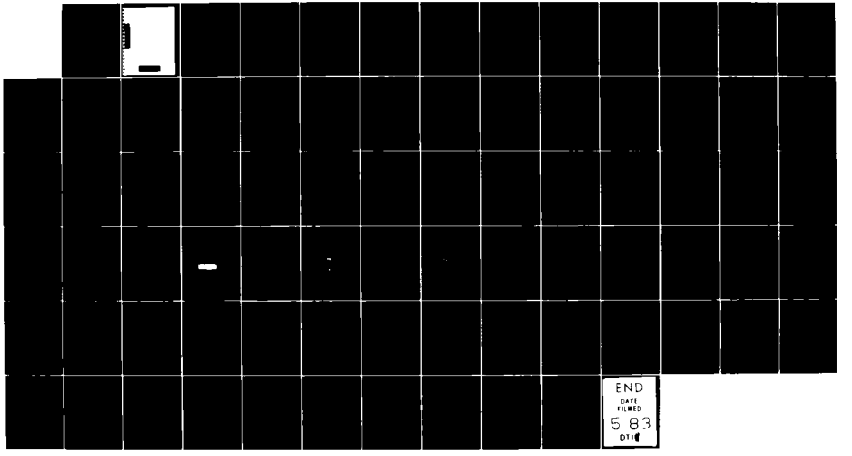

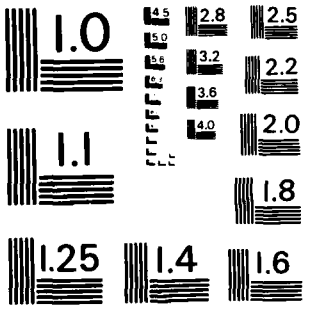

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REPORT NUMBER VIII

CONTROL OF HEMOTROPIC DISEASES OF DOGS

Annual Progress Report

Miodrag Ristic

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FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

A

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I. SUMMARY OF PROGRESS DURING 1977

Answers to 3 research objectives have been sought during the previous year of support. These objectives were (A) the effect of low level tetracycline treatment initiated during the early phase of infection with Ehrlichia canis on the outcome of the disease, (B) definition and characterization of the function and specificity of humoral factors related to target cell injury in canine ehrlichiosis, (C) assessment of the effect of nonspecific immunopotentiation on protection against E. canis infection in the German shepherd dog.

A. The effect of low level tetracycline treatment initiated during the early phase of infection with E. canis on the outcome of the disease.

Earlier studies showed that an oral continuous administration of tetracycline can be used therapeutically and prophylactically to control canine ehrlichiosis. A combination of such treatment with the use of indirect fluorescent antibody (IFA) test to monitor immune responses has produced excellent disease control results among military dogs in endemic areas. Based upon these earlier studies, several well-controlled experiments have been initiated by the U.S. Army Medical Unit in Kuala Lumpur, Malaysia, in collaboration with this laboratory to determine more exactly the effect of such treatment initiated during the various phases of the disease. The ultimate goal of these investigations is to develop a standard operational procedure (SOP) for control of ehrlichiosis in military dogs in endemic areas.

Experiments conducted during the past year were concerned with application of low level tetracycline (3 mg/lb/day), starting

at 7 and 14 days after infection and continuing for 30 days. The treatment was successful regardless of the time (7 or 14 days after infection) when administration of tetracycline was initiated. Signs of the disease in dogs started on tetracycline 14 days post-inoculation disappeared rapidly after treatment was initiated. Results based on subinoculation of blood from infected to normal dogs during and after treatment showed that the treatment cleared all dogs of infection. Dogs cleared of infection, however, showed no protective immunity upon reinfection. Strong but transitory antibody responses were noted in all infected dogs regardless of when tetracycline therapy was initiated. Dogs which were reinfected at 60 days after tetracycline treatment was discontinued, redeveloped antibody titers to E. canis. At 6 weeks after reinfection, the antibody titers, however, did not exceed titers measured in these animals as a result of the primary infection.

The isolation of E. canis from a dog in Negri Sembilan, Peninsular Malaysia, afforded an opportunity to study properties of the local strain. Mixed breeds of adult dogs were inoculated intravenously with this E. canis isolant. Inoculated dogs developed signs of the disease which included fever, weight loss, lymphodenopathy, corneal opacity, and pancytopenia. Of 3 dogs that died during the course of the study, one died with severe pancytopenia 78 days post-inoculation, and hemorrhagic lesions were prominant in numerous organs. All inoculated dogs developed strong antibody titers to antigen prepared from a U.S. isolant of E. canis, indicating cross-serologic relationship between 2 isolants.

During the past year, 873 sera of dogs belonging primarily to the U.S. Armed Forces were examined for antibodies to E. canis by the IFA test; a total of 278 of these dogs were positive.

Serologic examination for babesiosis using the IFA test was made on 214 dogs belonging to the U.S. Armed Forces and allied armies. A total of 151 of these dogs were positive.

B. Definition and characterization of the function and specificity of humoral factors related to target cell injury in canine ehrlichiosis.

Previous studies have implicated immunologic mechanisms in the pathogenesis of thrombocytopenia associated with canine ehrlichiosis. The Progress Report of 1976 described the presence of the platelet migration inhibition factor (PMIF) in the serum of dogs infected with E. canis. The presence and concentration of the PMIF was measured by the platelet migration inhibition test (PMIT).

Preliminary studies initiated in 1976 aimed at determining the nature and properties of the PMIF were continued during 1977. It was established that PMIF was contained in the 7S serum fraction purified by gel filtration and ion-exchange chromatography. Electrophoretic properties in immunoelectrophoresis and polyacrylamide gel electrophoresis tests and gel diffusion studies indicated that this was an IgG molecule. The fraction was resistant to 2-mercaptoethanol. Fractions isolated from normal or E. canis infected dogs were immunochemically identical, indicating that the inhibitor was not an E. canis antigen or a protein moiety

synthesized de novo but an immunoglobulin with measurable specificity for platelet membrane antigen, probably having some pathogenic implications. Chicken erythrocytes coated with platelet antigen and labelled with ^{51}Cr were specifically lysed by PMIF, thereby demonstrating the antigen-antibody nature of the reaction between platelets and PMIF.

Failure of the purified fraction to cause any in vivo effect is puzzling, however, it may be speculated that in vivo homeostatic mechanisms interferred with the expression of the PMI activity measurable under highly controlled and optimized in vitro conditions.

C. Immunopotentiation of dogs against tropical canine pancytopenia (canine ehrlichiosis) by means of inoculation with Bacille Calmette Guerin (BCG).

Preliminary studies have shown that German shepherd dogs which developed a non-severe syndrome of tropical canine pancytopenia (TCP) had responded to a higher degree in the leukocyte migration inhibition (LMI) test for cell mediated immunity (CMI) to E. canis than similar dogs which demonstrated a severe form of the disease.

It was postulated that immunologic competence of the cellular type plays a decisive role regarding the clinical outcome of TCP in German shepherd dogs. Three experiments were conducted to evaluate CMI responses in dogs inoculated with BCG and E. canis administered singly or in combination.

In the first experiment, German shepherd dogs were inoculated intramuscularly with a live E. [illegible] and LMT and blast transformation

(BT) tests were performed on a weekly basis; protein purified derivative (PPD) was used as antigen of choice to measure immune responses to BCG. These tests were useful in detecting the ability of dogs to respond to BCG.

In the second experiment, dogs were infected with E. canis. LMI and BT rests were performed on a weekly basis using cell culture-derived E. canis antigen. One of the 2 dogs failed to respond in either of the 2 tests for CMI. This animal died of the disease. The second dog responded in the LMI but not in the BT test. This dog developed a mild disease and recovered.

In the third experiment, animals were first inoculated with BCG and 7 days later infected with E. canis. Tests for CMI were performed on a weekly basis, using PPD and E. canis antigens, respectively. In this group, one dog responded to PPD in LMI but not in the BT test. This animal, however, failed to respond in either test to E. canis antigen. The dog developed a severe disease syndrome. The second dog of this group responded in LMI but not in BT tests to both PPD and E. canis antigens. This dog developed a mild form of the disease.

Results of the above studies suggest that German shepherd dogs able to generate CMI responses to E. canis are more likely to overcome the severe syndrome of TCP than those which fail to do so. The study needs to be repeated on a greater number of dogs to produce statistically significant results.

A peculiar aspect of TCP is that blood macrophages, and apparently other lymphatic elements, are affected by invasion, growth and development of E. canis. Thus, it is possible that the sequence of host cell-parasite interaction may bring about an impairment of the function of the immune system in general, which would result in a failure of infected dogs to mount a detectable CMI response. It is possible that use of E. canis of lower virulence would minimize this impairment of lymphatic function and enable infected dogs to respond more readily to CMI stimulation.

II. DETAILED PROGRESS REPORT

Research accomplished under Objective 1

A. Tetracycline Treatment

Results of tetracycline treatment started 7 days post-infection with E. canis are given in Table 1. Five dogs were inoculated intravenously with blood from an acutely infected E. canis carrier on April 12, 1977. Seven days post-inoculation each dog began receiving daily tetracycline treatment, administered in dosages of 3 mg/ml body weight, for a period of 30 days. Serum samples were obtained from each dog at approximate 7-day intervals from April 5, 1977 to June 23, 1977 for subsequent examination by the IFA test.

Preinoculation serum samples, obtained April 5, were negative for anti-E. canis antibodies in the IFA test. Within 21 days post-inoculation and 7 days after onset of tetracycline treatment, all dogs demonstrated a serologic conversion. In 4 of the 5 dogs

examined, antibody response was transitory. Antibody was detected in serum taken May 3 from dogs 87, 118, 129, and 130 and May 9 from dog 130. Dog 79 experienced a prolonged antibody response beginning 14 days post-inoculation. Although antibody persisted for 34 days, the antibody titer steadily declined from a high of 1:160 to 1:10 (Fig. 1). With the exception of dog 79, antibody was not detected in serum of any other animal collected following cessation of tetracycline treatment.

Verification of E. canis infection in dogs 79, 87, 118, 129, and 130 and the ability of these dogs to transmit the infection was determined by subinoculations of whole blood from treated dogs into susceptible dogs 3, 6, 28, and 68 days post-infection.

Four of the 5 dogs subinoculated April 15 and all of the dogs subinoculated April 19 developed clinical symptoms of E. canis and became serologically positive for antibodies to the disease. Dogs subinoculated May 10, during the course of tetracycline treatment, and June 18, following termination of treatment, did not develop clinical symptoms nor did they produce detectable antibody to the disease agent.

The results of tetracycline treatment started 14 days post-infection with E. canis are given in Table 2. The experimental method followed varies from that described for Table 1 in only 2 regards: tetracycline treatment was initiated 14 rather than 7 days post-inoculation and dogs were rechallenged 53 days after termination of treatment.

All dogs were serologically negative for anti-E. canis antibody prior to inoculation. Following inoculation, antibody

response occurred 7 days earlier in these dogs than in the dogs included in the previous experiment. Dog 88 became serologically positive 7 days post-inoculation, dogs 117, 119, and 131 within 14 days and dog 95 within 21 days. In contrast to the generally transitory response noted in Table 1, all dogs maintained a serologic response for at least 28 days and in one instance for as long as 49 days. During a brief period begining June 13 and extending to June 20, no antibody was detected in serum from any of the dogs. However, low titer antibody was again detected in 4 out of 5 dogs 78 days post-inoculation and 34 days following termination of tetracycline treatment.

Prior to termination of tetracycline treatment, antibody titers began to decline. Four days following end of treatment, titers increased slightly, then continued to decline. Fourteen days after reinfection, titers increased sharply and remained high during the remainder of the study (Fig. 2, 3).

Consistent with the results observed in experiment 1, blood taken from these dogs 21 days after onset of tetracycline treatment and 28 days after the initiation of treatment was not infectious when subinoculated into normal dogs.

B. Serology Performed on Military Dogs for U.S. Armed Forces.

During the past year, 878 sera of dogs belonging primarily to the U.S. Armed Forces were examined for antibodies to E. canis by the IFA test; a total of 278 of these dogs were positive.

Serologic examination for babesiosis using the IFA test was made on 214 dogs belonging to the U.S. Armed Forces and allied armies. A total of 151 of these dogs were positive.

Serologic studies conducted in cooperation with the U.S. Army Medical Research Unit, Malaysia, are given in Table 3.

Table 1: Serologic responses of dogs treated with tetracycline beginning 7 days post inoculation.

Dog	5 Apr	12 Apr	Start Tetracycline ↓ 19 Apr	26 Apr	3 May	9 May	16 May	19 May Stop Tetracycline ↓ 23 May	30 May	6 Jun	23 Jun
79	—	Inoc.	—	+ 1:160	+ 1:160	+ 1:80	+ 1:40	+ 1:20	+ 1:10	—	—
87	—	Inoc.	—	—	+	—	—	—	—	—	—
118	—	Inoc.	—	—	+	—	—	—	—	—	—
129	—	Inoc.	—	—	+	—	—	—	—	—	—
130	—	Inoc.	—	—	+	+	—	—	—	—	—

15 Apr Subinoculated

Dog	Sero.	Clin.
73	+	+
93	+	+
101	+	+
115	—	?
120	+	+

19 Apr Subinoculated

Dog	Sero.	Clin.
68	+	+
86	+	+
96	+	+
116	+	?
123	+	+

10 May Subinoculated

Dog	Sero.	Clin.
76	—	—
97	—	—
100	—	—
132	—	—
133	—	—

18 Jun Subinoculated

Dog	Sero.	Clin.
31	—	—
74	—	—
113	—	—
128	—	—
138	—	—
108	—	—

Sero.: Indicates positive (+) or negative (-) results for presence of antibody by IFA test.

Clin.: Indicates presence of symptoms (+), no observed symptoms(-), or questionable symptoms (?) of E. canis syndrome.

Table 2: Serologic responses of dogs treated with tetracycline beginning 14 days post inoculation.

Dog	5 Apr	12 Apr	19 Apr	26 Apr (Start Tetracycline)	3 May	9 May	16 May	23 May	30 May	6 Jun (19 May Stop Tetracycline)
88	—	Inoc.	+ 1:10	+ 1:320	+ 1:320	+ 1:320	+ 1:320	+ 1:160	+ 1:320	+ 1:160
95	—	Inoc.	—	—	—	—	+	+	+	—
117	—	Inoc.	—	+	+	—	+	+	—	—
119	—	Inoc.	—	+	+	—	—	—	—	—
131	—	Inoc.	—	+ 1:1280	+	+ 1:320	+	+ 1:10	+ 1:40	—

	13 Jun	20 Jun	29 Jun	18 Jul (Rechallenge)	25 Jul	1 Aug	8 Aug (17 May *Subinoculated dogs 70, 84, 108, 110, 134)	15 Aug	22 Aug	1 Sept
88	—	—	+ 1:80	+ 1:20	+ 1:10	+	+1:160	+	+	+ 1:640
95	—	—	—							
117	—	—	+	+	+	+	+	+	+	+
119	—	—	+	+	+	+	+	+	+	+
131	—	—	+	+ 1:20	+	+ 1:640	+	+ 1:640	+	+ 1:640

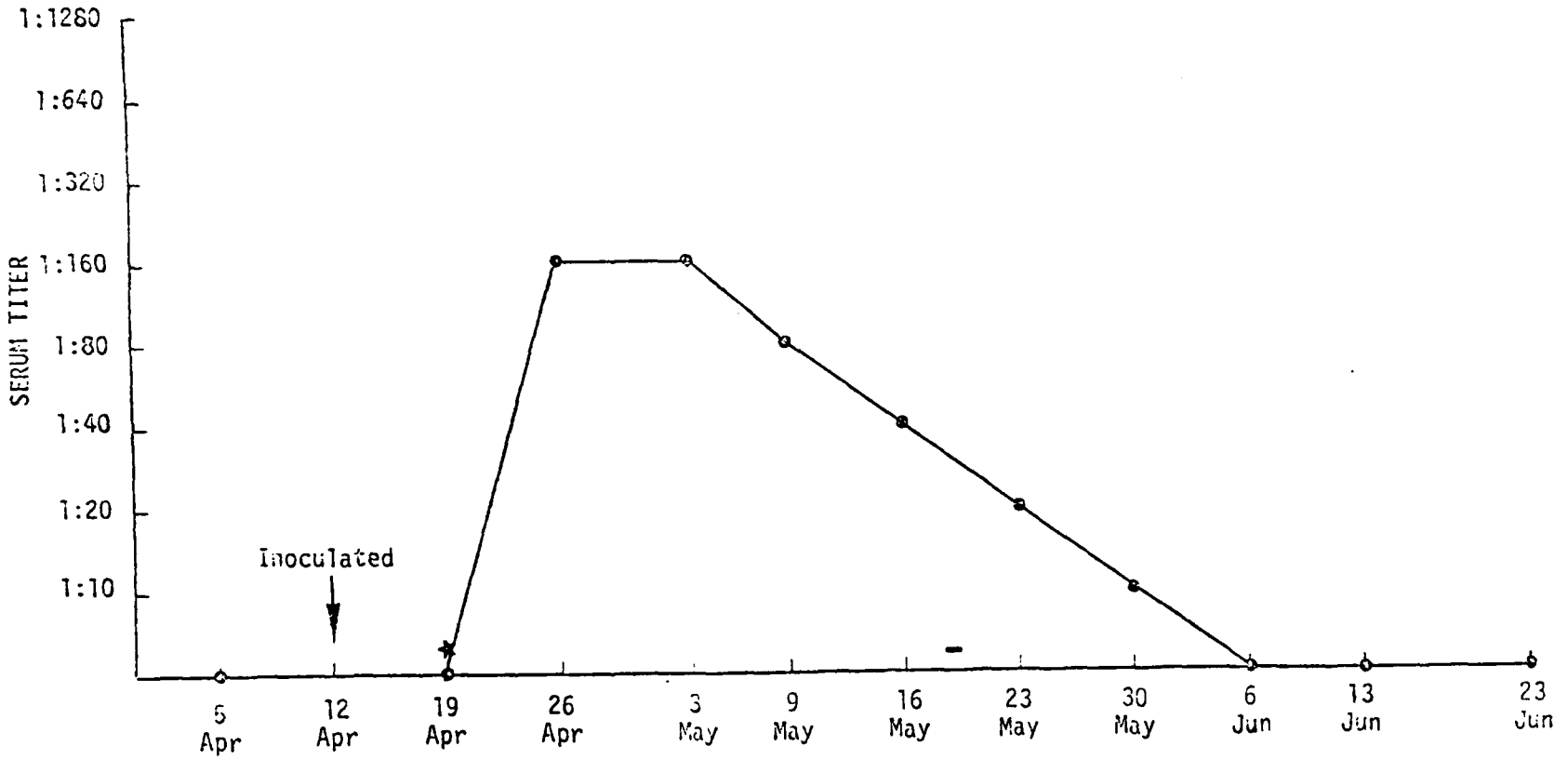
* 23 Jun Subinoculated dogs 104, 139, 140, 141, 146, 84, 97, 132, 133, 134

* None of the dogs subinoculated developed clinical symptoms of ehrlichiosis, nor did they produce antibody to the E. canis organism.

Table 3. Serologic studies conducted in cooperation with the U.S. Army Medical Research Unit, Malaysia.

Study	No. Sera Screened	No. Sera Positive	No. Sera Titered
Evaluation of occurrence of *Babesia canis* in the Malaysian dog population	195	132	--
Evaluation of Malaysian *E. canis* isolate - sera tested for:			
B. canis	19	19	--
E. canis	19	19	19
Effect of prophylactic tetracycline on *E. canis* infections	357	138	75

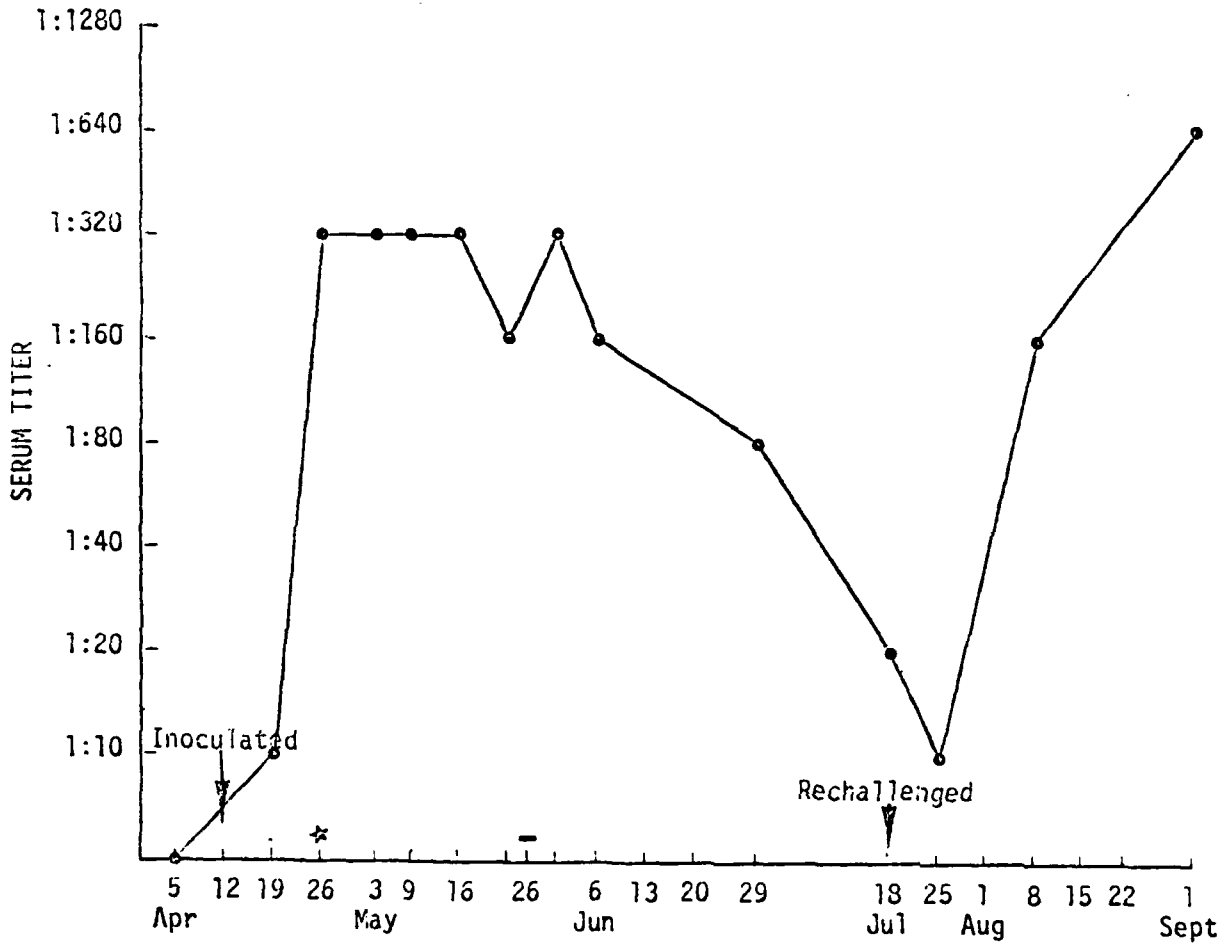
Figure 1. SERUM ANTIBODY TITRATION OF DOG 79 WHICH RECEIVED PROPHYLACTIC TETRACYCLINE TREATMENT BEGINNING 7 DAYS POST INOCULATION



✱ Start tetracycline (3 mg/lb)

– Stop tetracycline

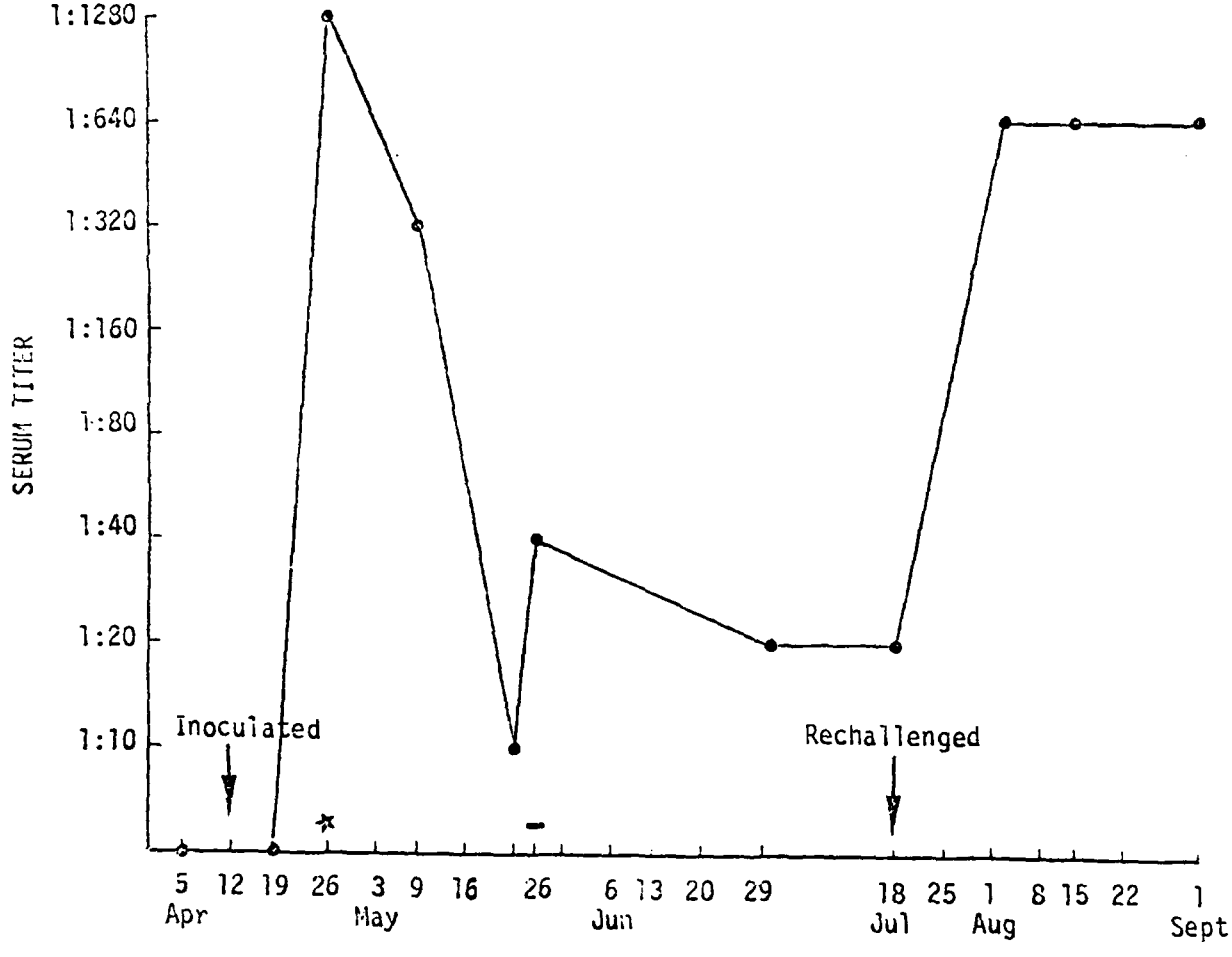
Figure 2. SERUM ANTIBODY TITRATION OF DOG 88 WHICH WAS TREATED WITH TETRACYCLINE 14 DAYS POST INOCULATION AND RECHALLENGED 53 DAYS AFTER TERMINATION OF PROPHYLACTIC TREATMENT



*Start tetracycline (3 mg/lb)

-Stop tetracycline

Figure 3. SERUM ANTIBODY TITRATION OF DOG 131 WHICH WAS TREATED WITH TETRACYCLINE 14 DAYS POST INOCULATION AND RECHALLENGED 53 DAYS AFTER TERMINATION OF PROPHYLACTIC TREATMENT



*Start tetracycline (3 mg/lb)

–Stop tetracycline

C. Study of Malaysian Ehrlichia canis Isolant.

In the 1960's canine ehrlichiosis with hemorrhagic manifestations accounted for the death of numerous military and privately owned dogs in Singapore[1,2] and Malaysia,[3] however, few studies of the disease in this country have been reported. The isolation of E. canis from a dog in Negri Sembilan, Peninsular Malaysia, afforded an opportunity to study the disease in experimentally infected, mixed breed dogs.

Materials and Methods

The isolate of E. canis used in this study was obtained from a pet bulldog in Seremban, Negri Sembilan. The dog had been sick, and during routine hematological examination, organisms were seen in mononuclear cells in Giemsa-stained blood smears. Using a syringe wetted with heparin containing 20,000 U.S.P. units per ml, 15 ml of blood were collected from the dog. The blood was transported to Kuala Lumpur on wet ice and was inoculated intravenously into a laboratory dog. The organism was maintained by serial passage in dogs.

The 9 dogs in the study were mixed breed, adult dogs weighing 25 to 40 pounds. With the exception of the dog inoculated with blood from the original case, each dog was inoculated intravenously with 10 ml of whole blood collected in ethylene diamine tetracetic acid (EDTA) from a dog acutely ill with the infection. Of 7 infected dogs studied, 4 were inoculated with blood from the third passage and one each with blood from the original case, first passage and second passage. Two dogs were used as

uninfected controls and were examined in the same manner as the infected dogs.

Clinical and hematologic examinations were made throughout the study and sera were collected monthly for examination in the IFA test.

Results

Although some dogs had rectal temperatures of 103° F or greater as early as 9 days post-inoculation, all dogs were febrile at 12 days. Pyrexia persisted for approximately 2 weeks. Thereafter, the mean temperature returned to near normal.

The thrombocyte count dropped sharply from over 300,000/mm^3 at time of inoculation to below 40,000/mm^3 by day 12. The mean thrombocyte count remained under 100,000/mm^3 throughout most of the experimental period. On numerous occasions thrombocyte counts of 5,000/mm^3 or less were recorded.

The hematocrit fell to below 30% by day 12 post-inoculation, remained constant near 30% through day 72 post inoculation, and then gradually increased to above 40% at approximately 120 days post-inoculation. The lowest hematocrit recorded was one value of 10% on day 77 post-inoculation in a dog which succumbed to the disease. Hemoglobin levels followed the same pattern as that of the hematocrit.

The white cell count dropped from a mean of 18,000/mm^3 on day of inoculation to a low of 6100/mm^3 on day 97. Repetitive white cell counts of less than 5,000/mm^3 were recorded for 2 dogs 56-72 days post-inoculation.

Erythrocyte sedimentation rates (ESR) rose rapidly from a

mean of 2.33 mm/hr at time of inoculation to 34.57 mm/hr at 12 days post-inoculation. Six of the 7 dogs had an ESR of at least 40 mm/hr sometime during the experimental period. One dog maintained an ESR of 5 mm/hr or below except for day 19 when it reached 23 mm/hr. One dog which died with severe hemorrhagic lesions at 78 days post-inoculation had marked pancytopenia at time of death.

Weight loss of 5 to 7 pounds was noted in each dog with a gradual return to normal in surviving dogs. Swelling of prescapular and popliteal lymph nodes was observed as early as 3 weeks post-inoculation in 3 dogs and persisted for several months. Corneal opacity was observed in 2 dogs. In one dog corneal opacity became evident 3 weeks post-inoculation and persisted throughout the observation period. In the other dog corneal opacity appeared 6 weeks post-infection and persisted for 30 days.

Epistaxis, which is commonly seen in Alsatian dogs infected with E. canis, was not seen in these dogs; however, 2 dogs developed cutaneous petechial and ecchymotic hemorrhages at days 46 and 55 post-inoculation. The hemorrhages were most noticeable around the shoulders, neck, flanks and abdomen. The hemorrhages on 1 dog disappeared within 3 weeks after they were first noticed. The other dog died approximately 3 weeks after the hemorrhages were first seen and the hemorrhages were still prominent at the time of death. A necropsy examination on this dog revealed a generalized hemorrhagic condition. Hemorrhages were seen in the skin and subcutaneous tissues, mammary glands, lymph nodes, lungs,

heart, gastrointestinal tract, urinary bladder and pleura. The dog that recovered from the hemorrhagic condition later developed severe ascites and ventral abdominal and peripheral subcutaneous edema which was first noticed at day 120 post-inoculation. This condition progressed until the dog died on day 170. At necropsy approximately 1500 ml of pale yellow, slightly opaque fluid was removed from the abdominal cavity.

In addition to the 2 dogs described above that died at 78 and 170 days post-inoculation, one other dog died 38 days following infection. The only significant finding in this dog was ascites. A fourth dog became extremely emaciated and was killed 82 days post-inoculation. Gross necropsy examination of the latter dog revealed no significant lesions.

The uninfected dogs showed no signs of disease and hematological values remained unchanged.

Serological examination of sera collected at monthly intervals from experimentally infected dogs revealed that antibodies to E. canis were present one month post-inoculation and throughout the experimental period or until death. Antibody titers varied from 1:640 to 1:1280.

Morulae of E. canis in mononuclear cells in Giemsa stained blood smears were seen on occasion but were generally very difficult to find.

Summary

Mixed breed, adult dogs were inoculated intravenously with Ehrlichia canis isolated from a dog in Peninsular Malaysia. Signs of disease included fever, weight loss, lymphadenopathy,

corneal opacity, and pancytopenia. Of 3 dogs that died during the course of the study, one died with severe pancytopenia 78 days post-inoculation, and hemorrhagic lesions were prominent in numerous organs. All inoculated dogs developed strong antibody titers to E. canis. Since the test used an E. canis isolant from the U.S., it is evident that the Malaysian and the latter isolant are serologically closely related.

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3. Wilkins, J. H., Bowden, R. S. T. and Wilkinson, G. T.: A new canine disease syndrome. Vet. Rec., 81 (1967): 57-58.

Research accomplished under Objective 2

A. Immunopatnologic Features of Canine Ehrlichiosis, or Tropical Canine Pancytopenia (TCP).

1. General Information

A. Previous and Current Immunopathologic Findings. Previous studies[1,2,3,4] have implicated immunologic mechanisms in the pathogenesis of the thrombocytopenia associated with canine ehrlichiosis. It was also demonstrated that during the course of the disease, platelet survival time was shortened, presumably due to increased platelet destruction.[3] The work reported here is concerned with the in vivo and in vitro characterization of the platelet migration inhibition factor (PMIF) measured by the platelet migration inhibition test (PMIT).[3] It was demonstrated that the factor was an immunoglobulin predominantly of the IgG class. The factor was found in highest concentration during the acute phase of the disease. Absorption studies using normal platelets, normal monocytes and monocytes infected with Ehrlichia canis established that the PMIF was distinct from the specific anti-Ehrlichia canis antibody measured in the IFA test. Using a cytotoxicity assay based on the ^{51}Cr-release from chicken erythrocytes to which platelet extracts had been chemically coupled, it was shown that the interaction between PMIF and the platelet membrane was a genuine antigen-antibody reaction. In vivo studies remained inconclusive, presumably due to complex homeostatic mechanisms interferring with the inhibitory activity demonstrable under highly controlled in vitro conditions.

b. Canine ehrlichiosis, or tropical canine pancytopenia (TCP) is an arthropod-borne disease of the canidae. It presents a variety of individual and breed-related symptomatology ranging from mild to severe. Typically, there is a pancytopenia mainly affecting the platelets. In adult dogs, the mild form is common whereas puppies suffer a more severe disease.[5] More recently Buhles et al.[1] divided the disease into different phases. The acute phase occurs in all breeds during the first 2 to 4 weeks of infection and is characterized by mild signs and transient pancytopenia. In beagles (which exemplify breed resistance) the disease progresses into a stable mild chronic state. German shepherds (which exemplify breed susceptibility) tend to have severe pancytopenia, hemorrhage and peripheral edema due to secondary infection. This form of the disease has been termed severe chronic and is often terminal due to excessive bleeding.[6]

The pathogenesis of the pancytopenia has received extensive attention but remains poorly understood. Burghen et al.[4] suggested that the hypergammaglobulinemia may be related to a state of hypersensitivity related to an autoimmune process induced by the infection. This hypothesis has been supported by Hildebrandt et al.[8] and Ristic et al.[9] It has been suggested[7] that anti-Ehrlichia antibody may contribute to the pathogenesis of the disease, particularly in relation to maintenance of the carrier state. Thrombokinetic studies by Smith et al.[3] and radioisotope

studies on the bone marrow by Buhles et al.[1] have implicated depression or malfunction of the bone marrow precursor cells as the primary disorder leading to thrombocytopenia. Thrombocytopenia may also be the result of excessive platelet destruction, perhaps related in part to the immune response.[3]

The purpose of the present investigation is to further examine the nature of one of the effector mechanisms directed against platelets as target cells in canine ehrlichiosis.

B. Characterization of the Humoral Factors Related to Platelet Injury in TCP

1. Studies on the specificity of PMIF. Differentiation of anti-E. canis activity from anti-host cell histocompatibility antigen activity. Comparative kinetic studies were carried out to examine the PMIF response in needle-infected dogs compared with dogs infected by tick transmission.
2. Tick-transmission of ehrlichiosis. A beagle dog shown to be free of ehrlichiosis was infected by needle inoculation of 10 ml of blood from an E. canis carrier dog. During the acute phase of the disease, as determined by platelet counts, 200 normal Rhipicephalus sanguineus nymphs were introduced into tick capsules placed on the dog's neck. The nymphs became replete after 7 days, were removed and kept in a tick incubator[a] under controlled conditions of temperature and humidity where they were allowed to moult into adults.

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[a]Precision Scientific Corporation, New York, NY.

Tick capsules were placed on a normal dog. Twenty infected adult male and 20 female ticks were fed on the dog for 7 days. Platelet counts, percent platelet migration inhibition (PMI) and indirect fluorescent atnibody (IFA) titers were determined weekly throughout the 4-week surveillance period.

3. Induction of ehrlichiosis by needle inoculation. A dog shown to be free from ehrlichiosis was inoculated intravenously with 10 ml of anticoagulated blood (1 part of 20% sodium citrate to 20 parts blood) from an E. canis-carrier dog. Platelet counts, PMI activity and IFA titers were monitored weekly for 50 days. To control for anti-blood group response, a normal dog was inoculated intravenously with 10 ml of whole normal dog blood in 20% sodium citrate as anticoagulant.

4. Preparation of platelets. The method described for use with canine platelets is a modification of the technique of Duquesnoy et al.[10] Twenty ml of blood was drawn in 2 ml of 20% sodium citrate solution. The blood was centrifuged at 400 to 500 g for 10 minutes in siliconized polycarbonate tubes at room temperature. The upper 3/4 of the platelet-rich plasma (PRP) layer was removed and centrifuged at 1000 g for 10 minutes. The platelet pellet was resuspended in 0.1 ml of supernate designated platelet poor plasma (PPP). The remaining PPP was inactivated at 56° C for 30 minutes and lipids removed by centrifugation at 7000 g for 30 minutes. One normal dog was used as a standard source of

platelets which, in all cases, were determined nonreactive with preinfection serum collected from experimental dogs.

5. Platelet migration inhibition (PMI) test. A medium[10] was prepared by mixing 3 parts of MEM with 2 parts of autologous PPP and 200 units of penicillin-streptomycin. Two-tenths of a ml of inactivated test serum was added to 0.2 ml of a suspension of platelets containing 1 to 2 x 10^8 platelets per ml. The mixture was incubated at room temperature for 1 hour. Micro-capillary tubes (25λ) were filled with the platelet-serum suspension and heat sealed. After centrifugation at 1000 g for 3 minutes, the capillary tubes were cut just above the interface between packed platelets and supernatant solution. The closed end of the stumps were embedded into silicone-grease in the center of a 25 mm cover slip which served as the floor of the migration chamber.[b] The migration chambers were closed with a second cover slip adhered by the silicone-grease. The space between the 2 cover slips were filled with the medium and the entry portals sealed with hot paraffin wax. All tests were carried out in triplicate and each chamber contained at least 3 stumps. The chambers were incubated at 37° C for 16 hours. The areas of migration were measured using a light microscope with a calibrated ocular micrometer at 40 x magnification. The mean percent migration was

[b]University of Illinois, Urbana, IL.

calculated relative to the degree of inhibition induced by preinoculation serum.

6. Absorption procedure. Serum aliquots (0.5 ml) were mixed with 2 x 10^6 platelets in a pellet to avoid dilution effect. The mixture was incubated at 37° C for 1 hour and centrifuged at 450 x g for 5 minutes to separate the serum. The serum was diluted 1:10 in phosphate buffered saline (PBS) before IFA or PMI tests were performed. For absorptions using monocyte cultures, normal and infected monocytes were cultured as described by Nyindo et al.[11] Serum aliquots (0.5 ml) were incubated with 2 x 10^6 monocytes at 37° C for 1 hour and centrifuged at 450 g for 5 minutes.

   The IFA test was performed as previously described.[9]

7. Identification of serum fraction with PMI activity using molecular sieve chromatography. Sephadex G-200 was equilibrated in 0.1 M Tris-HCl buffer for 24 hours. After equilibration and calibration for 19S (IgM) 7s (IgG) and 5-6S (albumin), 2 ml sample fractions were applied to a 100 x 2.5 cm column. The absorbance was monitored and recorded at 280 nm using a VA-5 automatic UV monitor-recorder.[c] Tubes related to each peak were pooled and the eluate dialyzed against 0.15 M NaCl to restore isotonicity. The fractions representing each peak were then concentrated by lyophilization to the original sample volume (2 ml) and tested in the PMI test for activity.

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[c]International Scientific Co., New York, NY.

8. Purification of IgG. The PMI activity seemed to be associated with the IgG fraction of serum collected from dogs in the acute stage of ehrlichiosis. The method of Steinbuch and Audran[12] for IgG purification was then used with subsequent separation of molecular sub-species by anion exchange chromatography. One-hundred ml of serum was mixed with 200 ml of 0.96M acetate buffer pH 4.0. Caprylic acid (6.8 g) was added dropwise with vigorous stirring for 1 hour. The mixture was then centrifuged at 700 g for 10 minutes. The precipitate was discarded and the supernatant further purified on an anion exchange column equilibrated with 0.01 M phosphate buffer. A 25 x 2.5 cm Sephadex A-50 column was used and samples containing 100 mg of protein were applied and eluted with 0.01 M phosphate buffer. The fractions were tested in the PMI test for inhibition activity.

9. Immunoelectrophoresis. A modification of the technique of Scheidegger[13] was used to determine the purity of fractions obtained from molecular sieve and ion exchange chromatography. One percent agarose was prepared in tris-barbital high-resolution buffer[d] diluted in water (75 parts water to 25 parts buffer). The electrophoresis chamber[d] was filled with undiluted buffer. Slides were precoated with a thin layer of agarose and covered with 4 ml of agarose. Wells were cut and filled with fractionated sample, electrophoresis was carried out at 300 volts (constant voltage) for 1 1/2 hours, and troughs were filled with serum from rabbits hyperimmunized against canine serum components. Slides were allowed to incubate in humidity chambers overnight. Slides were examined before and after staining with Coomasie blue.

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[d]Gelman Instruemtn Corp., Ann Arbor, MI.

10. Polyacrylamide gel electrophoresis of chromatographic fractions associated with PMI activity. A 30% solution of polyacrylamide was prepared by adding 30 gm of acrylamide[e] to 100 ml of distilled water. The solution was filtered through a Whatman #1 filter and stored at 4° C.

A gel was prepared by mixing 15 ml of 30% acrylamide gel, 8.4 ml of 2.0 M Tris-HCl buffer pH 8.8 and 20.85 ml of distilled water. To this mixture 0.3 ml of ammonium persulfate (APS) (100 mg/ml) and 12.5 lambda of N', N', N'-tetramethylenethylenediamine (TEMED) was added. The stacking gel was prepared by mixing 2 ml of 30% acrylamide, 1.9 ml of distilled water, 2 ml of Tris-HCl buffer, 20 lambda of APS, 10 lambda TEMED and 5 ml of 1% agarose. Sample buffer was prepared by mixing 2 ml of Tris-HCl buffer and 2 ml of 20% sucarose and 0.5 ml of bromophenol blue. Ten lambda of chromatographic sample with proven PMI activity was mixed with 10 lambda of sample buffer and carefully layered into the sample wells. Samples from normal dogs eluting in the same region were similarly analyzed. The chambers were filled with Tris-Hcl buffer. Stacking was performed at room temperature at 20 mA at constant voltage and further electrophoresis was carried out at 4° C at the rate of 50 mA constant voltage. Electrophoresis was continued until the bromophenol blue indicator reached the bottom of the slab.

The gel slab was removed and stained in Coomasie blue (0.2% Coomasie brilliant blue, 50% methanol, 7% acetic acid).

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[e]Bio-Rad Laboratories, Richmond, CA.

Destaining was accomplished in 10% acetic acid. A denatured sample was prepared in the same manner with the addition of 0.25% 2-mercaptoethanol (2-ME), and 012% sodium dodecyl sulfate to the sample. Gel slabs were scanned at 540 nM on an Ortec scanner.[f]

11. Demonstration of binding properties of platelet migration inhibition factor. Cytotoxicity measurement using ^{51}Cr release assay. The method of Voigtmann et al.[14] was used with slight modification to prepare soluble antigen from canine platelets. Platelets from citrated canine blood were separated as described above. To a suspension of 1 x 10^9 platelets in 1 ml of PPP was added 5 ml of 0.3 M KCl solution. After gentle shaking, the mixture was incubated at 56° C for 1 hour. The suspension was agitated every 15 minutes. The lysate was then filtered through a Whatmann #1 filter, divided into 0.2 ml aliquots, stored at -20° C until required for use. The protein concentration was adjusted to 10 mg/ml before use.

Chicken erythrocytes were collected from adult white Leghorn fowls. Five ml of blood was collected in a test tube containing sodium oxalate as anti-coagulant. The cells were extensively washed with PBS and finally resuspended in RPMI 1640 to 2 x 10^8 cells per ml.

The procedure of Johnson et al.[15] was used to couple the platelet antigen (PAG) to chicken erythrocytes. To 0.3 ml of PAG solution containing a total of 3 mg of protein was added

[f]Ortec Corporation, New York, NY.

10^8 erythrocytes in 0.1 ml. One ml of 0.1% 1-ethyl-3 (3-diethylaminopropyl) carbodiimide hydrochloride in PBS was added. The mixture was left at room temperature for 1 hour with shaking every 15 minutes. The cells were washed 3 times in 1% heat-inactivated rabbit serum. The coated cells were resuspended in 1% normal rabbit serum to a concentration of 2×10^8 cells per ml.

The technique of Perlmann and Perlmann[16] was used to label the antigen-coated erythrocytes with ^{51}Cr and cytotoxicity test was carried out as described above for the monocytotoxicity test.

The activities of PMIF were also assayed in the indirect hemagglutination (IHA) and hemolytic tests by a micro technique. Purified PMIF was resuspended in PBS at a concentration of 10 mg/ml. Dilutions of PMIF in 0.025 ml were added to each well. An equal volume of PAG-coated chicken erythrocytes was added and the mixture was incubated at room temperature for 1 hour before reading the hemagglutination pattern. The end point of the hemagglutination was recorded as the highest dilution showing a distinct button of cells. After the reading, 0.025 ml of guinea pig complement was added and incubation continued for 30 minutes. The hemolytic titer was scored as the highest dilution of reactant that lysed 50% of a standard suspension of erythrocytes (2×10^6 per well).

12. In vivo testing of the effect of platelet migration inhibition factor. Serum collected from a dog during the acute stage of ehrlichiosis was separated by column chromatography using

Sephadex G-200. Each fraction was tested in the PMI test for evidence of anti-platelet activity. A control preparation was made from preinoculation serum collected from the same dog. The fractions were analyzed at 280 nm and concentration determined. Two dogs were inoculated intravenously with 22.2 gm and 31.2 gm, respectively, of the fraction which had the most PMI activity. Two control dogs were inoculated with 36.65 gm and 100 gm, respectively, prepared from preinoculation dog serum. The dose of the inhibitor was computed such that the concentrations of normal serum proteins were elevated 100% in an effort to artificially induce hypergammaglobulinemia. Platelet levels were monitored every hour for the first 5 hours. Platelet counts were also determined at 12, 24 and 48 hours post-inoculation and thereafter every 2 days for 2 weeks.

C. Results

Gel-filtration and ion-exchange chromatography demonstrated that the platelet migration inhibitor resided in the IgG fraction of inhibitory serum. This was confirmed by immunoelectrophoresis (Fig. 1) and polyacrylamide gel electrophoresis of whole serum and isolated IgG fractions (Fig. 2,3). There was no detectable difference in electrophoretic mobility or number of protein bands between fractions isolated from normal dog serum and those obtained from immune serum (Fig. 4.5). Antiserum prepared against inhibitory canine serum failed to reveal any abnormal component in serum of infected dogs causing inhibition of migration of normal platelets.

Chicken erythrocytes coated with inhibitory IgG were positive

in the hemagglutination test and in the ^{51}Cr release assay for cellular damage. The reaction was specific for platelet antigen (Table 1) and the inhibitor was 2-ME resistant.

Absorption of inhibitory serum with normal platelets removed inhibitory activity but did not affect the IFA titer (Table 2a). Conversely, absorption of the same serum with E. canis infected monocytes removed the IFA activity without a significant effect on the PMIT (Table 26). Incubation of inhibitory serum with normal monocytes did not affect either of the reactions.

A dog infected by ticks infected with E. canis developed significant levels of PMIF between 5 and 9 days post-infection and the activity persisted until day 32 post-infection (PI) (Table 3). The onset of inhibitory activity coincided with diminishing levels of platelet count. The control dog inoculated with isologous normal canine blood maintained normal platelet counts and showed only base line levels of PMI activity.

D. Summary and Discussion

An investigation was conducted to determine the nature of the platelet migration inhibition factor (PMIF) associated with tropical canine pancytopenia (TCP). The PMIF was contained in the 7S fraction purified by gel filtration and ion-exchange chromatography. Electrophoretic properties in immunoelectrophoresis and polyacrylamide gel electrophoresis confirmed that this was an IgG molecule. Further, it was shown that the fraction was resistant to 2-mercaptoethanol. Fractions isolated from normal or E. canis-infected dogs were immunochemically identical, indicating that the inhibitor was not an

E. canis antigen or a protein moiety synthesized *de novo* but an immunoglobulin with measurable specificity for platelet membrane antigen and probably having some pathogenic implications.

Using a cytotoxicity assay based upon the ^{51}Cr-release from chicken erythrocytes to which platelet extracts had been chemically coupled, it was shown that the interaction between PMIF and the platelet membrane was a genuine antigen-antibody reaction.

The failure of the purified fraction to cause any *in vivo* effect is puzzling. However, it may be speculated that *in vivo* homeostatic mechanisms interferred with the expression of the PMI activity measurable under highly controlled and optimized *in vitro* conditions.

Table 1. ^{51}Cr release assay, hemagglutination (HA), and hemolytic (HL) tests of PMIF on chicken erythrocytes coated with solubilized platelet antigen.

Effector	^{51}Cr % released	HA NO 2-ME[a]	HL + 2-ME	HA NO 2-ME	HL + 2-ME	UE
Normal dog IgG NO C[b]	7.6	0	0	0	0	0
Normal dog IgG + C	8.1	±	±	±	0	0
Immune dog IgG NO C	10.24	±	0	0	0	0
Immune dog IgG + C	44.1	±	1:4*	1:2	1:8	0

a = 2-mercaptoethanol

b = complement

* = highest dilution of 100 mg/ml solution giving a positive reaction.

± or + = weak or strong positive with undiluted test material.

UE = uncoated chicken erythrocytes.

Table 2a. Results of the effect of absorption of IFA and PMIT positive dog serum, with normal canine platelets at room temperature.

Test	Before Absorption	After Absorption
IFA	1:320[a]	1:320
PMIT	60%[b]	14.5%

[a]Mean IFA titer. Each sample was absorbed in triplicate aliquots.
[b]Mean percent platelet migration inhibition activity relative to normal preinoculation dog serum.

Table 2b. Effect of absorption* of IFA and PMIT positive dog serum with normal and E. canis infected canine monocytes.

Test	Normal Canine Monocytes		E. canis Infected Canine Monocytes	
	Before Absorption	After Absorption	Before Absorption	After Absorption
IFA	1:320	1:320	1:320	1:40
PMIT	60%	54.3%	60%	59.1%

*Results are expressed as means of triplicate absorptions on the sample.

Table 3. Development of PMIF, in relation to platelet levels in dog #150 (tick-infected with E. canis), and dog #132 inoculated with 10 ml of normal canine blood.

DAYS PI	Dog #150 PTLT x10-3	PMI %	IFA	Dog #132 PTLT x10-3	PMI %	IFA
0	320	5	0	289	6.2	0
3	298	7.5	0	300	5.9	0
5	321	15	0	292	ND	0
9	300	36	1:20	280	7.2	0
12	240	27	1:20	283	6.8	0
14	270	22	1:80	290	5.5	0
25	280	ND	1:60	321	6.4	0
29	340	40	1:640	297	5.6	0
32	314	19.5	1:640	283	5.5	0

Table 4. Platelet levels x 10^3 per mm^3 in dogs inoculated with platelet migration inhibitor and those inoculated with pre-inoculation IgG preparation.

	Inoculum			
	Immune	IgG	Normal	IgG
Dog No.	654	50	661	B6
Time PI				
10 hrs	257	295	432	397
0	247	310	419	390
1	238	342	382	417
2	251	321	398	430
3	260	290	411	427
4	279	352	451	386
5	245	286	429	400
2 days	231	300	407	421
4	281	336	410	459
6	270	314	423	383
8	261	349	398	371
10	245	309	381	428
12	233	298	420	403
14	250	270	394	417

Figure 1. Immunoelectrophoretic pattern of:

(1) whole normal canine serum
(2) whole immune canine serum
(3) isolated platelet inhibitor both IgG_1 and IgG_2 were evident
(4) whole immune canine serum

Figure 2. Polyacrylamide SDS slab-gel electrophoresis of normal canine IgG (ND), and immune canine IgG (ID).

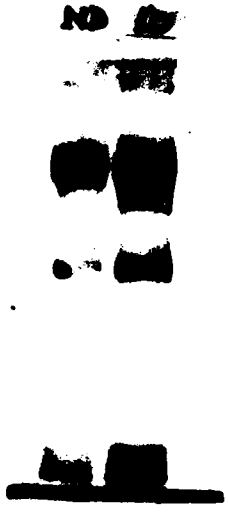


Figure 3. Polyacrylamide slab gel electrophoresis under non-denaturing conditions.
Left: Normal canine IgG (ND) and immune canine IgG (ID)
Right: Whole normal canine serum (ND)
Whole immune canine serum (ID)

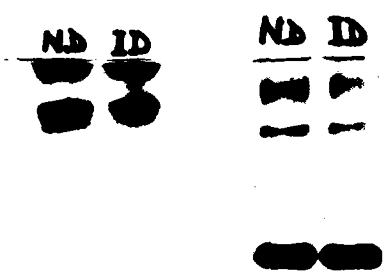
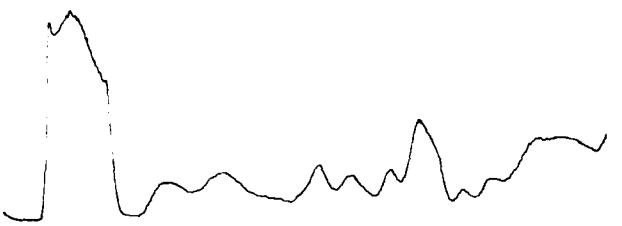

ND ID
ND ID

Figure 4. A densitometric scannine at 540 nM of non-denaturing polyacrylamide gel electrophoresis runs:
Top: whole normal canine serum
Bottom: immune whole canine serum



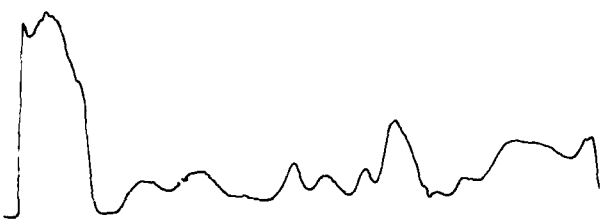
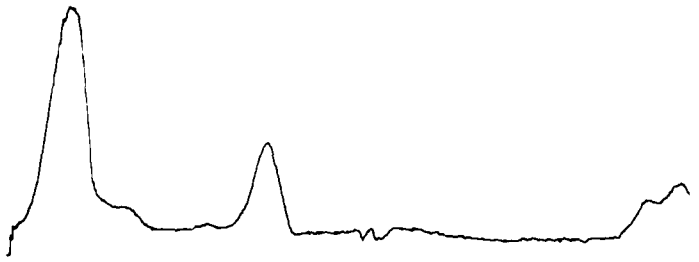
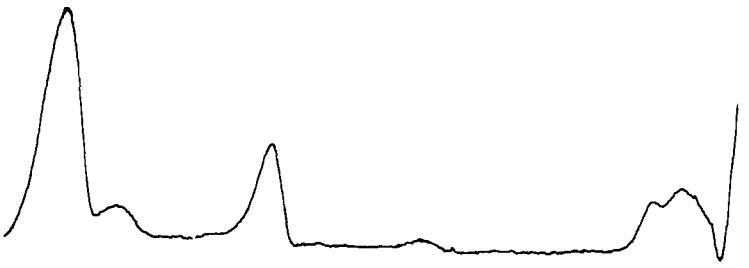


Figure 5. A densitometric scanning at 540 nM of SDS polyacrylamide gel electrophoresis runs:
Top: normal canine IgG
Bottom: immune canine IgG





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Research accomplished under Objective 3

A. Cell-Mediated Immunity and Protection in TCP

German shepherd dogs experimentally infected with Ehrlichia canis developed a severe hemorrhagic syndrome but experimentally infected beagles did not.[1] It was shown[2] that chronically ill German shepherd dogs developed marked thrombocytopenia, leukopenia, as well as hemorrhages and peripheral edema and at necropsy they showed massive internal hemorrhages, evidence of secondary bacterial infections and generalized perivascular plasmacytosis.

Hypergammaglobulinemia[3] and specific antibody for E. canis have been demonstrated. It was suggested[4] that CMI mechanisms may play an important role in the prevention of the disease since animals that showed a higher degree of CMI response as demonstrated in the leukocyte migration inhibition test developed a mild form of TCP. Thus it was postulated that immunologic competence of the cellular type plays a decisive role with reference to the clinical outcome of TCP in German shepherd dogs.

Three experiments were conducted to evaluate CMI responses in dogs inoculated with BCG and E. canis administered singly or in combination.

Six German shepherd dogs were used. In the first experiment 2 dogs received BCG only; 2 dogs of the second experiment were infected with E. canis; and the 2 dogs of the third experiment were first inoculated with BCG and 7 days later infected with E. canis. Cell mediated immune responses were monitored by use of the leukocyte migration inhibition (LMI) and the lymphocyte transformation (LT) tests. Results of these tests were correlated with clinical and hematologic findings.

B. Materials and Methods Used for Potentiation and Measurement of Cellular Immunity to E. canis

1. Experimental Animals

Adult Ehrlichia-free German shepherd dogs from 1 to 6 years of age were used in the experiments. All dogs were kept in environment controlled rooms. The animals were previously vaccinated against distemper, hepatitis, leptospirosis and rabies. Before use, each dog was examined clinically and wormed.

2. Antigens

Ehrlichia canis antigen (E. canis Ag) derived from monocyte cell culture was prepared by freezing-thawing techniques for use in the tests for CMI.

Normal dog leukocyte antigen (NDL Ag) was produced in the same way as E. canis Ag from uninfected cultures.

Protein purified derivative antigen (PPD) was obtained from a commercial source in lyophilized form. After reconstitution with sterile distilled water, aliquots of various concentrations were made and kept at - 70° C until used.

3. Organisms

Bacille Calmette Guerine (BCG) was obtained from the University of Illinois Medical Center* in lyophilized form. It was used immediately after reconstitution following the recommended dose for vaccination.

4. Parameters

Temperature, hematocrit and sedimentation rates were recorded at intervals. Determinations of red blood cell, white blood cell and platelet counts were also made. Weekly serum samples were obtained for the IFA technique.

*Lot IL 74 (S) 73; U of I., 903 West Adams St., Chicago, IL 60607.

5. Leukocyte migration inhibition test.

For leukocyte migration inhibition test (LMIT), 25 ml of blood was collected from the jugular vein into a 30 ml sterile disposable syringe that contained 2.5 ml of 20% Na citrate sterile solution as anti-coagulant. The blood was transferred to a 50 ml sterile siliconized plastic tube containing 15 ml of a sterile 9% Ficoll-50% Hypaque solution (28-10 parts) and centrifuged at 400 g for 80 minutes at 4° C. The middle layer that contained most of the white cell elements was decanted and mixed with Hank's balanced salt solution (HBSS) pH 7.2 and centrifuged twice at 700 g for 30 minutes at 4° C.

The leukoc pellet was resuspended in 0.3 ml of RPMI 1640 medium pH 7.2 and trypan blue stain for cell viability and concentration of cells was made. One-tenth of the ml of the desired cell concentration was mixed with the same volume of either RPMI 1640 medium, E. canis Ag dilution, or PPD at optimal concentration. The cell suspensions were incubated at 38° C for 30 minutes, then distributed in capillary tubes. Capillary tubes were centrifuged at 400 g for 10 minutes at room temperature, cut slightly below the cell pellet-medium interphase and placed in sets of 3 in specially designed migration chambers. Migration chambers were filled with adequate concentrations of either RPMI 1640 media, E. canis Ag dilution or PPD in RPMI 1640 medium, incubated at 38° C for 12-18 hours and area of migration was read in a graduated ocular.

6. Blast transformation tests.

Blood was drained by venipuncture from the jugular vein into a 50 ml sterile disposable syringe; 20% Na citrate sterile solution was used as anticoagulant at a rate of 1 ml of anticoagulant per 10 ml of whole blood. The blood was immediately transferred to a 50 ml sterile glass tube which was rolled over its axis for 5-10 minutes at 38° C. Twenty-five ml of blood was transferred to 50 ml sterile siliconized plastic tubes that contained 15 ml of a solution made of 9% Ficoll-50% Hypaque (28-10 parts). Tubes were centrifuged at 400 g for 85 minutes at 4° C; the middle layer was carefully removed and transferred to a 50 ml sterile siliconized plastic tube, mixed with HBSS and centrifuged twice at 700 g for 30 minutes at 4° C; the pellet was resuspended in 10 ml of RPMI 1640 medium, supplemented with 10% normal dog serum previously inactivated at 56° C for 30 minutes. Trypan blue stain for cell viability and concentration was performed and cell suspension adjusted to 5×10^6 cells/ml. One ml of cell suspension was then transferred to culture tubes. Different sets of tubes were treated with E. canis Ag dilution, PPD dilution, NDL Ag dilution, concavalin A and one test remained as untreated control. Cell suspensions were incubated at 38° C, 5% CO_2 in humid environment for 96 hours; all cultures were made in triplicate Five-tenths μCi of 2-^{14}C hymidine was added to each tube and incubated for 18 additional hours. Cell suspensions were centrifuged at 1000 g for 60 minutes at 4° C, washed with physiological saline solution, precipitated with 10% trichloracetic acid solution, centrifuged at 1000 g for 20 minutes at 4° C and supernatant was discarded. Two ml of ice cold pure methanol was added to each tube and centrifuged again

at 1000 g for 20 minutes at 4° C and supernatant was discarded. Tubes were allowed to dry overnight at 38° C.

Five-tenths ml of nuclear Chicago solubilizer was added to each tube and incubated at 50° C for 2 hours. Liquid scintillation cocktail (16.0 gms PPO per gallon of toulene) was added to each tube, shaken and transferred to disposable liquid scintillation vials, placed in counter at least 24 hours before counting. Results are reported as leukocyte stimulation index

C. Results

Experiment 1. Figure 1 shows the response to LMI test of dog GS #3 inoculated with BCG on day 0; by day 10 there was a detectable response; on day 38, reached the highest level at 55.1% inhibition. Table 1 shows response of dog GS #3 in the blast transformation test, to a mitogen (concavalin A) and PPD specific antigen with detectable response to BCG on day 7 after inoculation being the highest index of leukocyte stimulation on day 63 after inoculation. Figure 2 shows the percentage of migration-inhibition of dog GS #8 which was inoculated with BCG on day 0 with a detectable response on day 3 and the highest on day 24. Table 2 includes the results of blast transformation test using PPD as antigen expressed as leukocyte stimulation index, being the highest on day 28.

Experiment 2. The response of dog GS #5 is shown in LMIT is shown in Figure 3. It is noticeable that the percentage did not pass over the base line previous to inoculation. In fact, the animal died on day 55 after showing apathy and emaciation during the last week of life. Three days before death the animal developed epistaxis. Table 3 shows the index of stimulation of

the same dog when examined in the blast transformation test. Dog GS #6 showed a slightly higher LMIT response over base line by day 4, the highest on day 46 after inoculation (Fig. 4).

Data from the blast transformation test of dog GS #6 are presented in Table 4.

Experiment 3. In this experiment, 2 dogs were inoculated with BCG intramuscularly and exposed 7 days later with E. canis. Figure 5 shows the results of the LMIT to both antigens, PPD and E. canis, used in testing dog GS #7. In this test, the earliest response above base line for PPD was on day 38, and the highest on day 66. With regard to E. canis, the first response over the base line was detected on day 52 after inoculation with BCG (45th day after infection with E. canis) and the highest on day 66 (59th day after E. canis exposure). Table 5 presents the indices obtained in the blast transformation test for dog GS #7. Results of dog GS #10 for the LMI test are expressed in Figure 6, in which activity for PPD is first demonstrated on day 10 after inoculation with BCG and the highest response on day 38 after inoculation with BCG. Responses for E. canis Ag never went over base line. In Table 6, indices for blast transformation for dog GS #10 are presented.

D. Discussion and Summary

The leukocyte migration inhibition (LMI), as well as blast transformation (BT) tests, represent useful tools in detection of in vitro activity related to cell-mediated immune (CMI) mechanisms. Their advantage is that animals are not sensitized previous to inoculation as in the case of delayed cutaneous hypersensitivity.

At the time the experiments were designed, published information on detection of CMI in dogs was insufficient, thus a functional test to demonstrate such responses was developed. Bacille Calmette Guerin (BCG) was chosen as a sensitizing agent due to its known ability to stimulate delayed-type hypersensitive (DTH) reactions. Protein purified derivative (PPD), originally produced for skin reactivity tests, has been widely used as antigen of choice in many in vitro tests for CMI and therefore was chosen for this experimental design. The availability of these immunogens and specific antigens also accounted for their use in the previously described experiments.

Results obtained in the blast transformation tests were to demonstrate the ability of sensitized lymphocytes to react at a particular moment to a specific antigen. The differences in the indices measured are considered within expected limits of reaction. A positive reaction or ability to respond was estimated, based upon base line information rather than figures alone. Since the experiments were designed to evaluate responses on a weekly basis, early detections were not intended unless they were part of the protocol. The expression of indices in the BT test for concavalin A (Con-A) was used as a point of comparison for the specific response to a particular antigen as well as an assessing tool for the test system. The BCG dose at the moment of inoculation was the one recommended by the producer as standard vaccination for human beings. The route of inoculation for BCG was deeply intramuscular. Induration at the inoculation site was detected after

24 hours; redness and sore skin developed in one of the dogs (GS #3) by 48 hours. Lesions healed within 2 weeks by repeated cleaning with sterile physiological saline. No other substances were applied to the skin lesions. The characteristic of BCG for recruiting lymphocytes in the regional lymph node before they can exert any systemic action may explain the fashion in which LMI test reactions developed; with the highest level of activity between 3-5 weeks, then decreasing by 6 to 7 weeks post-inoculation. The size and extent of lesions developed in dog GS #3 may account for a faster and more extensive systemic infection that led to detection of activity after 70 days post-inoculation.

Failure of dogs GS #5 and #6 to respond in the BT test may be interpreted as follows: Macrophages are needed for a normal induction of lymphocyte blast transformation. In TCP, monocytes (blood macrophages) are the host cell for parasitic activity exerted by E. canis. It is possible that lymphocyte-macrophage interaction may be impaired but this phenomenon has not been measured. The indices for detection of specific T-cell mitogenic activity induced by Con A dropped after inoculation with E. canis. This may be an indication that lymphocyte transformation is suppressed. In the case of dog GS #5, this immunosuppression may have led to the death of the animal. In the case of dogs GS #5 and #6, a decrease in the index of blast transformation is noticed 2 weeks post-inoculation. The latter animal recovered after experiencing a mild form of the disease. It is possible that in the time between inoculation and impairment or suppression of the

immune system, some T-cells were sensitized. Although they were not able to show activity in the BT test, they showed a tendency to react in the LMI test. This was not the case for dog GS #5 since activity detected by this test never exceeded the predetermined base line.

Dogs inoculated with BCG and E. canis were able to react to a mitogen (Con A) in the BT test for a longer period of time than dogs that had been inoculated with E. canis alone. Dog GS #10 reacted as long as 3 weeks post-inoculation (PI) and dog GS #7 as long as 5 weeks PI. This phenomenon may be assumed to be caused by potentiation by BCG. Dog GS #7 was able to react to PPD at the beginning and end of the experiment, which possibly indicates that between inoculation and infection, a sufficient population of T-cells were sensitized. With respect to dog GS #10, no activity to PPD was detected, probably due to the same phenomenon that occurred in dogs GS #5 and #6. Although apparently both dogs (GS #7 and #10) had an active immune system, reactivity for E. canis antigen was not detected, possibly because macrophage-lymphocyte interaction did not occur.

In the LMI test the response of GS #7 was of increasing activity throughout the experiment. Therefore, suppression of macrophage activity, if present, might not have affected sensitized cells. Dog GS #10 was able to react with PPD, probably because sensitization did occur, but also showed a transient type of response. Dog GS # 10 showed no detectable activity for E. canis, as in the case of dog GS #5 yet it survived, indicating that when

animals are immunocompetent, they may overcome the disease and recover. Final conclusions can be made regarding immunopotentiation with BCG using an experimental animal population of adequate size.

Table 1.

Blast transformation test. Dog GS #3, inoculated with BCG (9.m)

Day	Con A	PPD
0	2.08*	1.31
7	18.24	4.08
14	8.25	2.22
21	1.54	1.57
28	3.58	2.31
35	29.76	6.21
42	89.30	5.48
49	9.82	3.74
56	15.01	1.54
63	8.05	13.25
70	13.75	9.31

*Leukocyte stimulation index = $\frac{\text{mean treated cultures (triplicates)}}{\text{mean control cultures (triplicates}}$

Table 2. Blast transformation test. Dog GS #8, inoculated with BCG (9.m).

Day	Con A	PPD
-21	10.11*	1.72
0	1.61	1.02
+7	1.14	0.81
14	1.22	0.66
21	5.84	1.02
28	19.33	4.09
36	6.07	1.67
43	3.45	2.46
63	2.71	0.80
71	1.50	0.57

*LSI = $\frac{\text{mean treated cultures (triplicates)}}{\text{mean control calves (triplicates)}}$

Table 3. Blast transformation test. Dog GS # 5, inoculated with *Ehrlichia canis* (sc).

Day	Con A	E. canis
-14	8.66*	1.41
+ 7	2.06	0.97
14	1.14	0.99
21	0.86	0.70
28	1.12	1.14
35	0.89	1.10
42	1.29	0.94
49	0.90	0.92
55	dead	

$$\text{LSI} = \frac{\text{mean treated cultures (triplicates)}}{\text{mean treated cultures (triplicates)}}$$

Table 4. Blast transformation test. Dog GS #6, inocualted with Ehrlichia canis (sc).

Day	Con A	E. canis
-7	17.0*	1.02
0	33.92	2.06
+8	39.10	1.30
15	45.09	1.35
35	1.25	1.30
43	1.40	0.56
49	1.39	1.14

*LSI = $\frac{\text{mean treated cultures (triplicates)}}{\text{mean control cultures (triplicates)}}$

Table 5. Blast transformation test. Dog GS #7, inoculated with BCG (i.m.) and *Ehrlichia canis* (sc).

Dog	Con A	PPD	E. canis
-7	38.46*	2.00	1.52
0	24.66	1.56	1.97
+7	6.53	1.24	0.82
14	18.09	6.06	1.21
21	3.74	1.43	0.25
28	14.03	2.24	1.55
35	3.14	5.09	1.23
42	0.98	1.23	1.03
49	0.88	0.88	0.87
56	0.87	0.75	0.85
63	6.57	3.47	0.81
70	29.28	26.53	1.61

*LSI = $\frac{\text{mean treated cultures (triplicates)}}{\text{mean control cultures (triplicates)}}$

Table 6. Blast transformation test. Dog GS # 10, inoculated with BCG (i.m.) and Ehrlichia canis (sc).

Day	Con A	PPD	E. canis
-7	21.13*	1.69	2.27
7	9.32	0.73	0.81
14	11.78	1.30	1.62
21	4.27	1.59	0.90
28	0.98	0.98	0.80
35	9.94	0.77	0.99
42	4.48	1.61	1.48
49	1.90	1.08	0.99
56	1.27	0.72	0.83
63	2.45	1.35	1.36
73	6.01	1.41	2.93

*LSI = $\frac{\text{mean treated cultures (triplicate)}}{\text{mean control culture (triplicates)}}$

Figure 1

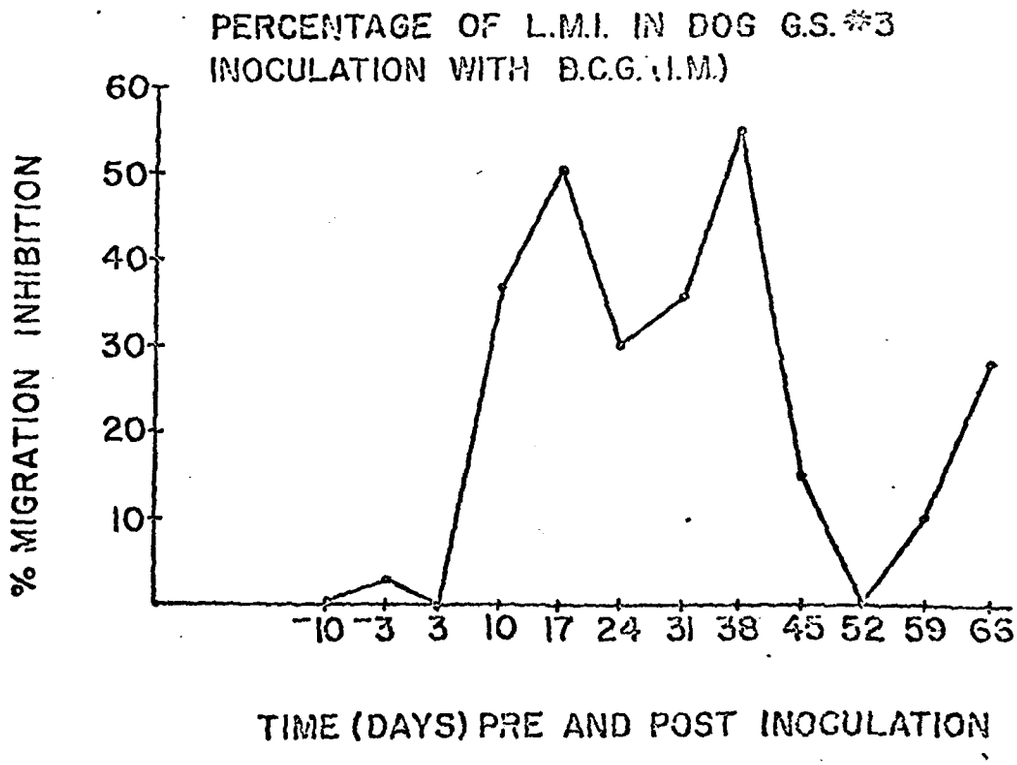

PERCENTAGE OF L.M.I. IN DOG G.S. #3
INOCULATION WITH B.C.G. (I.M.)
% MIGRATION INHIBITION
60
50
40
30
20
10
-10 -3 3 10 17 24 31 38 45 52 59 66
TIME (DAYS) PRE AND POST INOCULATION

Figure 2

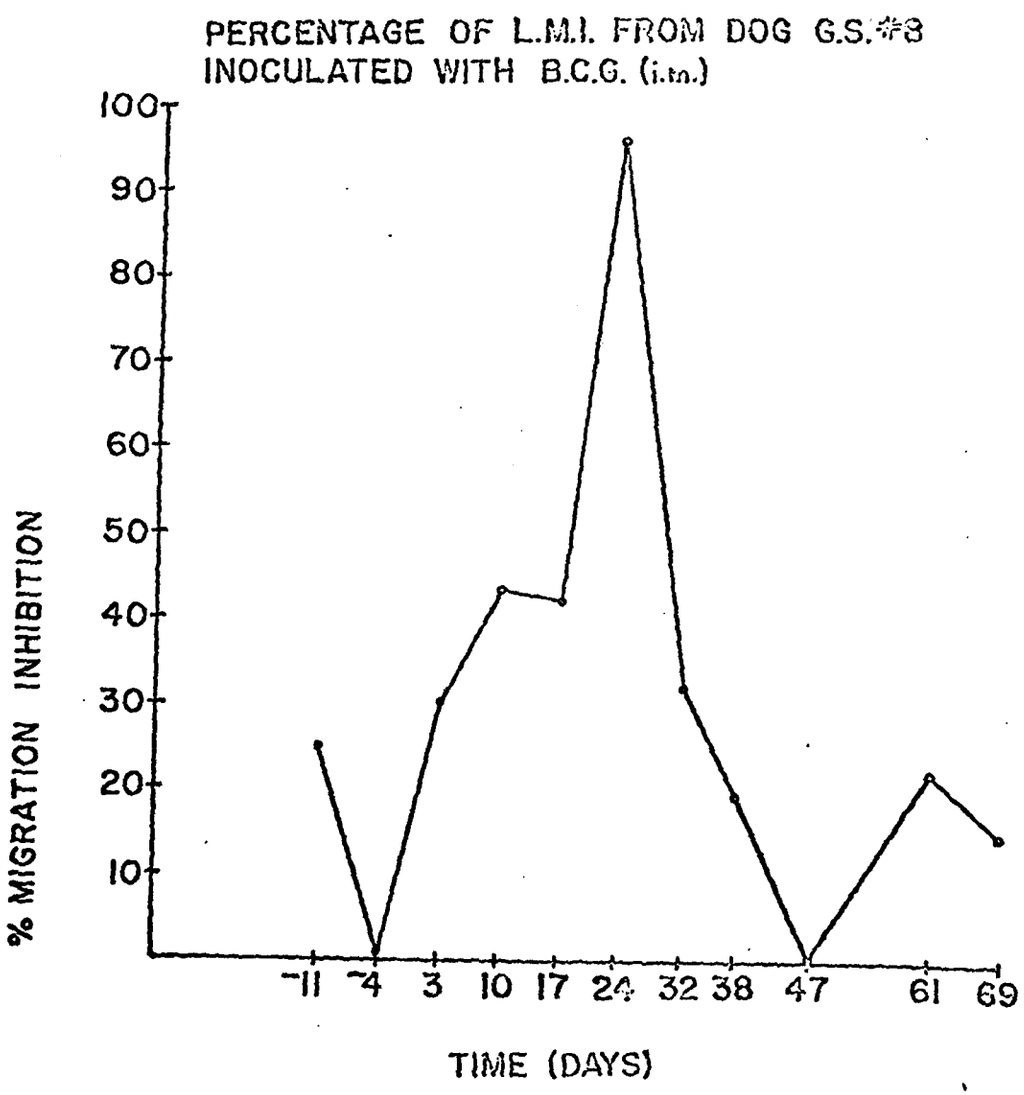

PERCENTAGE OF L.M.I. FROM DOG G.S. #3
INOCULATED WITH B.C.G. (i.tn.)
% MIGRATION INHIBITION
100
90
80
70
60
50
40
30
20
10
-11
-4
3
10
17
24
32
38
47
61
69
TIME (DAYS)

Figure 3

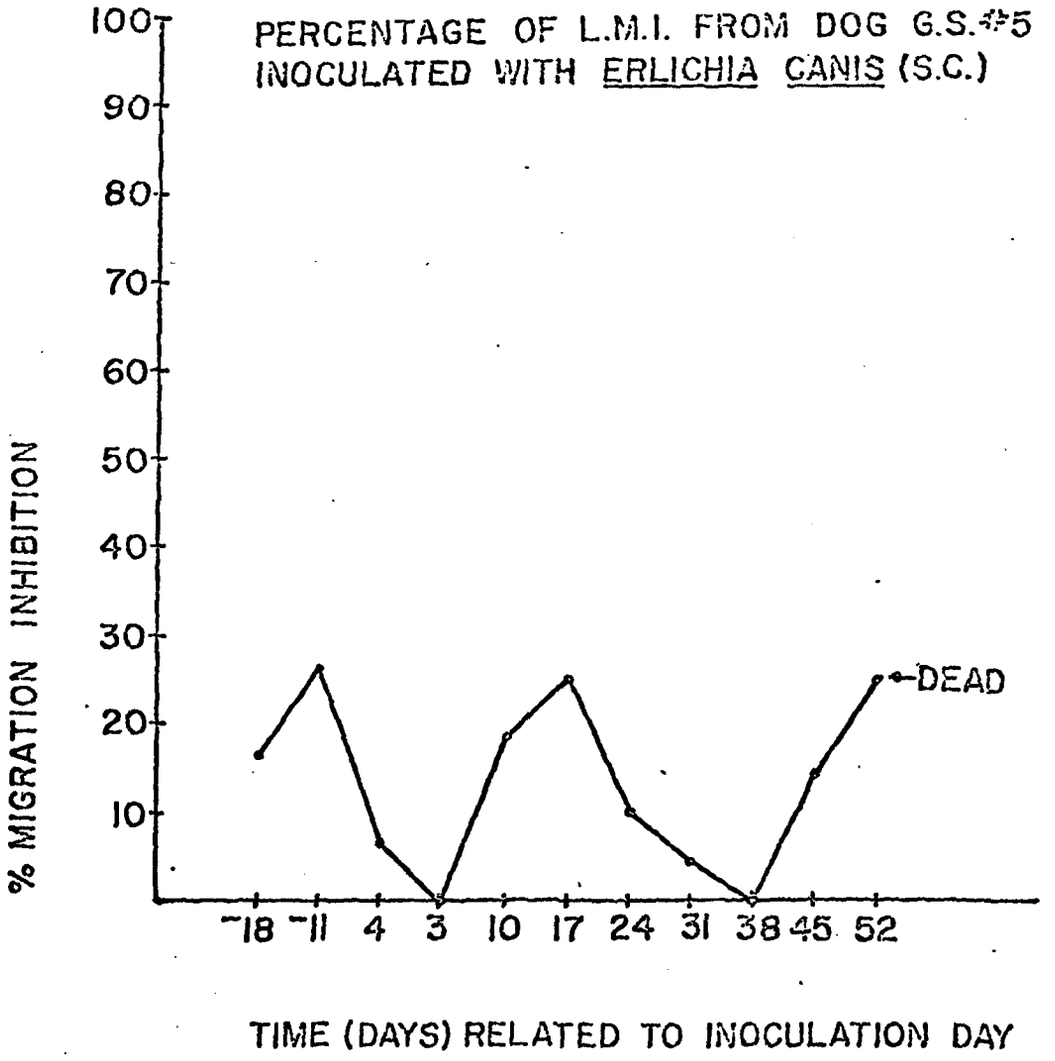

PERCENTAGE OF L.M.I. FROM DOG G.S.#5
INOCULATED WITH ERLICHIA CANIS (S.C.)
% MIGRATION INHIBITION
100
90
80
70
60
50
40
30
20
10
DEAD
-18 -11 4 3 10 17 24 31 38 45 52
TIME (DAYS) RELATED TO INOCULATION DAY

Figure 4

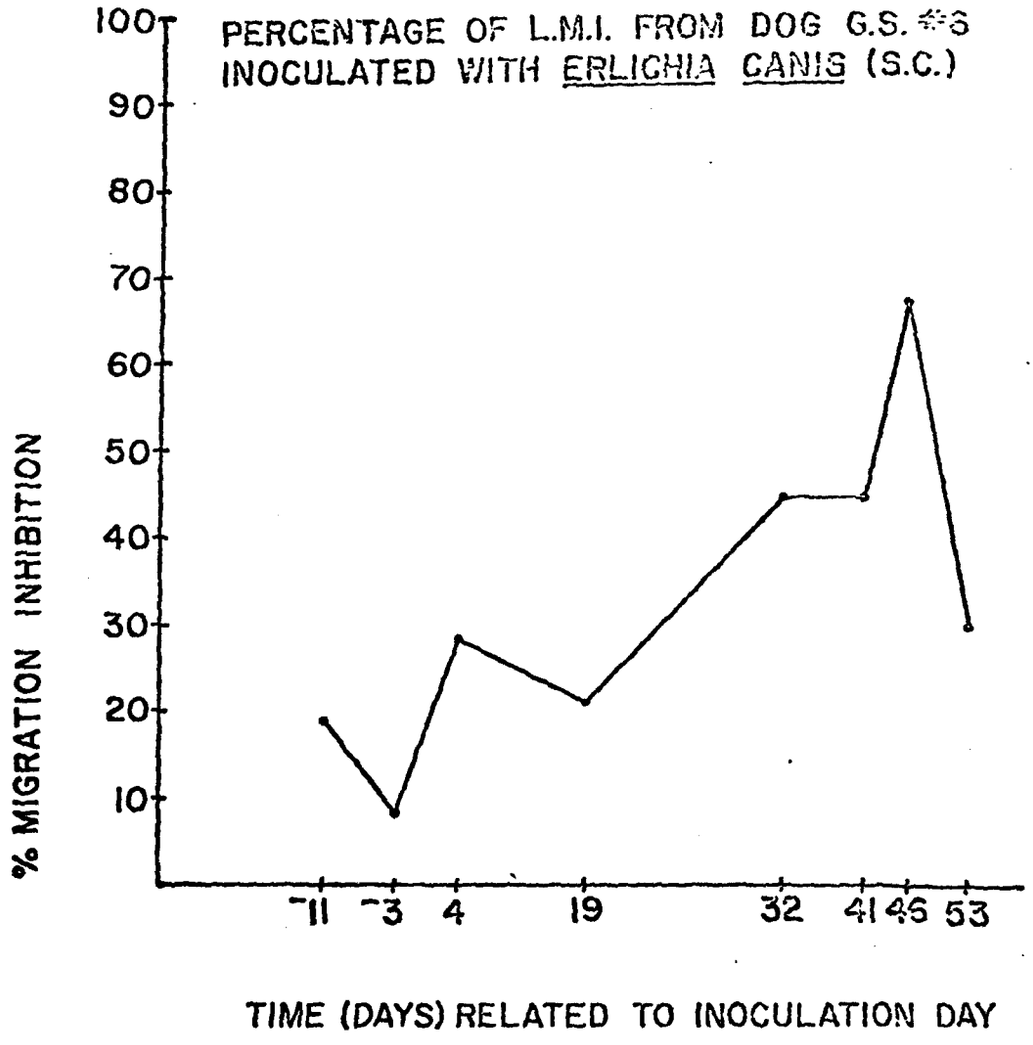

PERCENTAGE OF L.M.I. FROM DOG G.S. #6
INOCULATED WITH ERLICHIA CANIS (S.C.)
% MIGRATION INHIBITION
100
90
80
70
60
50
40
30
20
10
-11
-3
4
19
32
41
46
53
TIME (DAYS) RELATED TO INOCULATION DAY

Figure 5

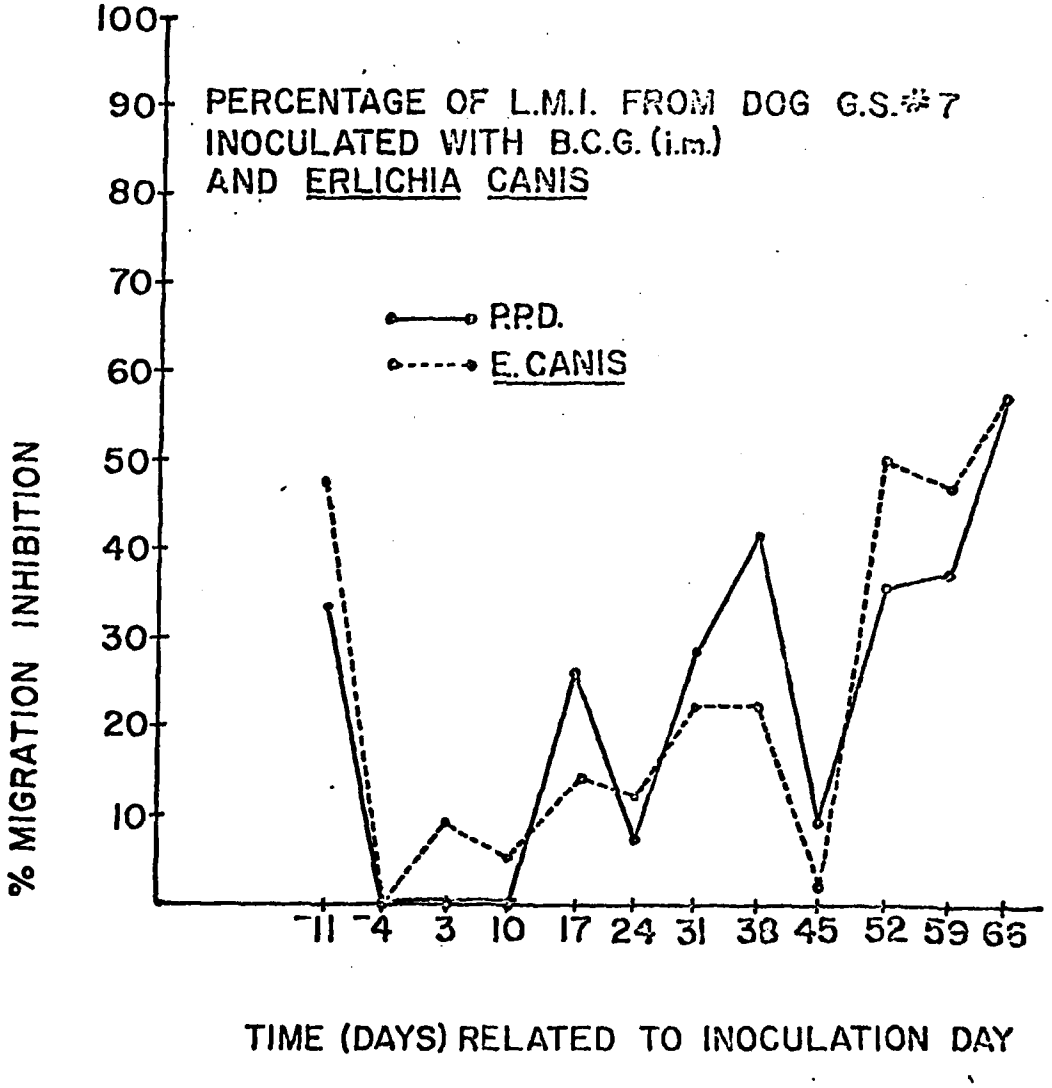
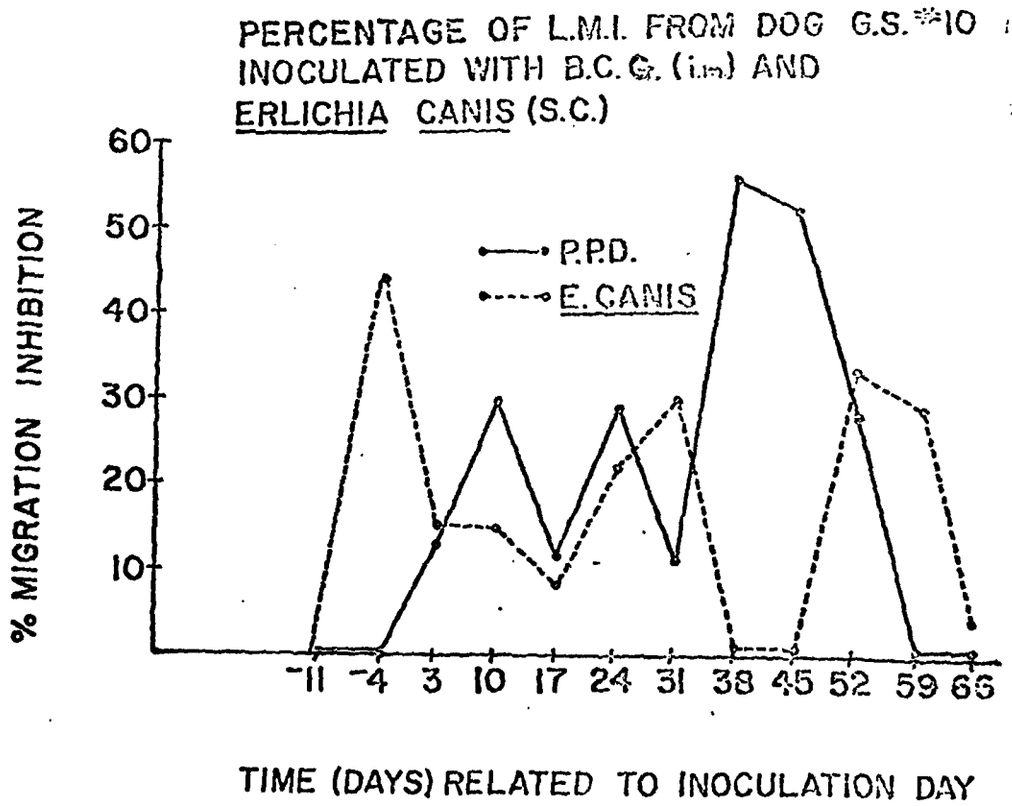

PERCENTAGE OF L.M.I. FROM DOG G.S.#7
INOCULATED WITH B.C.G. (i.m.)
AND ERLICHIA CANIS
P.P.D.
E. CANIS
% MIGRATION INHIBITION
100
90
80
70
60
50
40
30
20
10
-11
-4
3
10
17
24
31
38
45
52
59
66
TIME (DAYS) RELATED TO INOCULATION DAY

Figure 5


PERCENTAGE OF L.M.I. FROM DOG G.S. #10
INOCULATED WITH B.C.G. (i.m.) AND
ERLICHIA CANIS (S.C.)
% MIGRATION INHIBITION
60
50
40
30
20
10
P.P.D.
E. CANIS
-11 -4 3 10 17 24 31 38 45 52 59 66
TIME (DAYS) RELATED TO INOCULATION DAY

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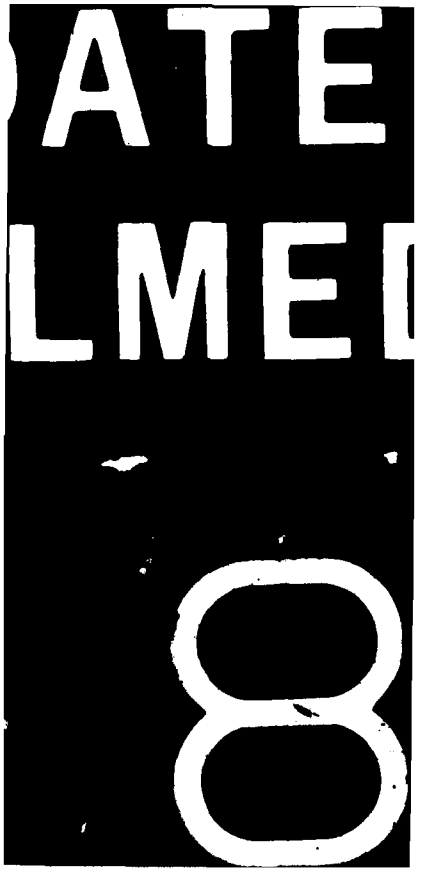
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